PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Atty. Docket No.: 01107.00134
Brad ST.CROIX	
Application Serial No.: 09/918,715	Group Art Unit: 1642
Filed: August 1, 2001	Examiner: C. Yaen
For: ENDOTHELIAL CELL EXPRESSION PATTERNS	

DECLARATION OF KENNETH W. KINZLER

Commissioner for Patents
Post Office Box 1450
Alexandria, Virginia 22313-1450

I, Kenneth W. Kinzler, declare:

1. I am a named co-inventor on the above-named application. I am a professor of oncology at The Johns Hopkins University School of Medicine in Baltimore, Maryland.

2. I have participated in further studies related to TEM 17, *i.e.*, the protein whose sequence is disclosed as SEQ ID NO:230 in the above-captioned application.

3. Our studies demonstrate that TEM17 protein is expressed in tumor endothelium but not in normal endothelium. Our studies further indicate that the extracellular domain of TEM17 protein binds to a protein known as cortactin. Cortactin is known to be involved in cell migration and the cytoskeleton. In addition, radiolabeled antibodies raised against TEM17 protein specifically label tumor tissues *in vivo*. The basis for these conclusions are described below.

4. We generated a monoclonal antibody that would specifically recognize TEM17.

The antibody, called IMT7, was generated against a peptide sequence (NNLSPKTKGTPVHLGTI) that resides in the extracellular domain of TEM17 (Fig. 1A). We also generated a rabbit polyclonal antibody against the entire extracellular domain of TEM17. Using 293 cells transfected with the gene encoding TEM17, we found that both the monoclonal and polyclonal antibodies were able to immunoprecipitate a product of ~85 kD (Fig. 1C). The electrophoretic behavior of this polypeptide was slower than that expected from the 54 kD predicted from its amino acid sequence. The decreased mobility was apparently due to glycosylation, as treatment with glycosidase reduced the apparent size by ~20 kD (Fig. 1C).

5. To determine if the expression of the TEM17 protein, like its mRNA, was elevated in colorectal tissues, we prepared protein extracts from normal colonic mucosa and colorectal tumors of five patients. Immunoblotting with TEM17 antibodies revealed elevated expression of the expected 85 kD product as well as additional 90 kD and 95 kD products in each of the tumor tissues tested (Figure 1D and data not shown). The reason for the two larger bands observed in each of the tumor samples is not clear, but could be due to alternative splicing, post-translational glycosylation, or ubiquitination. Although the relative abundance of each of the products in the 85/90/95 kD triplet varied between samples, all three were readily immunoprecipitated from tumor extracts with either the polyclonal or the monoclonal anti-TEM17 antibodies.

These data demonstrate that TEM17 protein is expressed in human tumor tissue and that its expression is elevated in tumor tissue relative to normal tissue.

6. To identify the cellular source of the TEM17 protein observed in the human tumor extracts, we performed a histological survey of various tumor types. As shown in Figure 2A, immunohistochemistry revealed a vessel-like pattern of staining in colorectal cancers. TEM17 staining was undetectable in the normal colonic mucosa. We observed a similar pattern

of staining of tumor vessels in esophagus, lung and bladder cancers (Fig. 2A and data not shown). To determine whether the endothelial cells were responsible for the vessel-like patterns of staining, we performed co-localization studies with an antibody to the pan-endothelial marker vWF. As shown in Figure 2B, TEM17 staining co-localized with vWF in tumor endothelium. Finally, we performed transmission electron microscopy using the same antibodies. TEM17 was predominantly expressed at the tight junctions between endothelial cells, although some expression at the luminal surface was also detected (Figure 2C).

These data confirm that TEM17 protein is expressed in tumor tissues and that it is differentially expressed in tumor and normal tissues.

7. To identify TEM17 binding partners, we constructed a fusion protein in which the extracellular domain of TEM17 was joined to alkaline phosphatase (AP) (AP-T7; Fig1A). The AP-TEM vector was transfected into mammalian 293 cells, and the secreted fusion proteins used to probe various tissue extracts using a modified western blotting assay. Protein extracts were resolved on denaturing gels, transferred to nitrocellulose, and blotted using AP-T7 fusion proteins. When various tissue extracts were tested in this assay using AP alone, no binding was detected. However, when probed with AP-T7, a prominent doublet of 80 and 85 kD was observed in every mouse and human tissue examined. Brain tissue appeared to be unique in that it contained a 75 kD product in addition to the 80/85 kD doublet (Figure 3A). We chose to use mouse brain for biochemical purification because the products recognized by the AP-T7 fusion protein was particularly abundant in this tissue.

Using the modified western blot assay as a screen, we biochemically fractionated and purified the 75 and 80 kD proteins using a strategy that involved tandem ion-exchange chromatography, affinity chromatography and preparative SDS-PAGE. When the 75 and 80 kD

bands were excised from the gel and analyzed by MS/MS Mass spectroscopy, both were independently identified as cortactin. Interestingly, others have shown that cortactin typically migrates as a doublet of 80/85 kd with the difference in size presumably due to phosphorylation. Furthermore, the additional ~75 kd product observed in brain tissue presumably represents a brain-specific alternative splice variant that harbors a deletion in the cortactin repeat region.

To determine if TEM17 could bind cortactin, the IMT7 monoclonal antibody was used to immunoprecipitate TEM17 from 293 cells expressing exogenous TEM17. A strong band of the expected size for endogenous cortactin was observed in these immunoprecipitates (Figure 3C). Importantly, endogenous TEM17 also co-precipitated with cortactin in lysates derived from rabbit tumor tissues, although only the 85 kD product of the triplet was observed (Figure 3D). Thus, cortactin appears to bind TEM17.

These data demonstrate a specific binding interaction between TEM17 and cortactin, and suggests a biological function for TEM17.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: August 05, 2004

Kenneth W. Kinzler

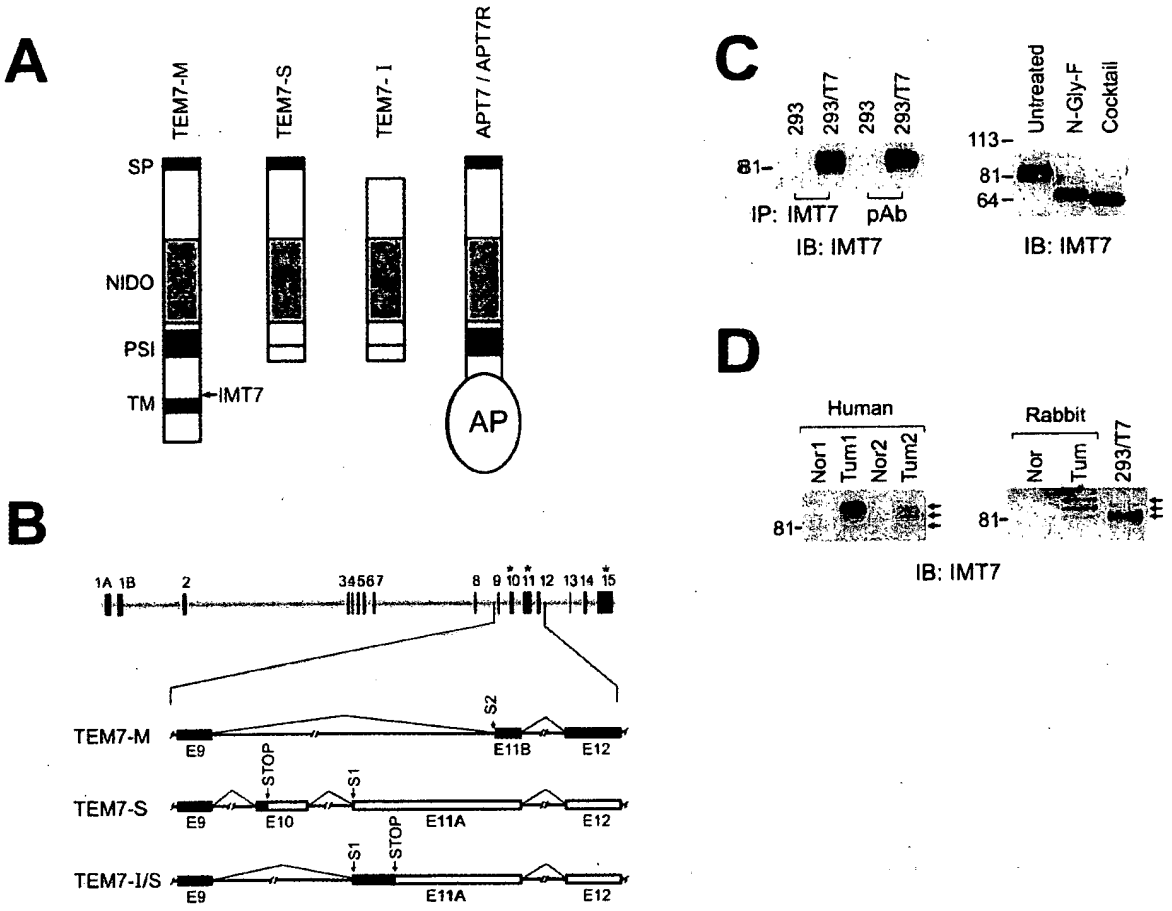


Figure 1, Nanda et al.

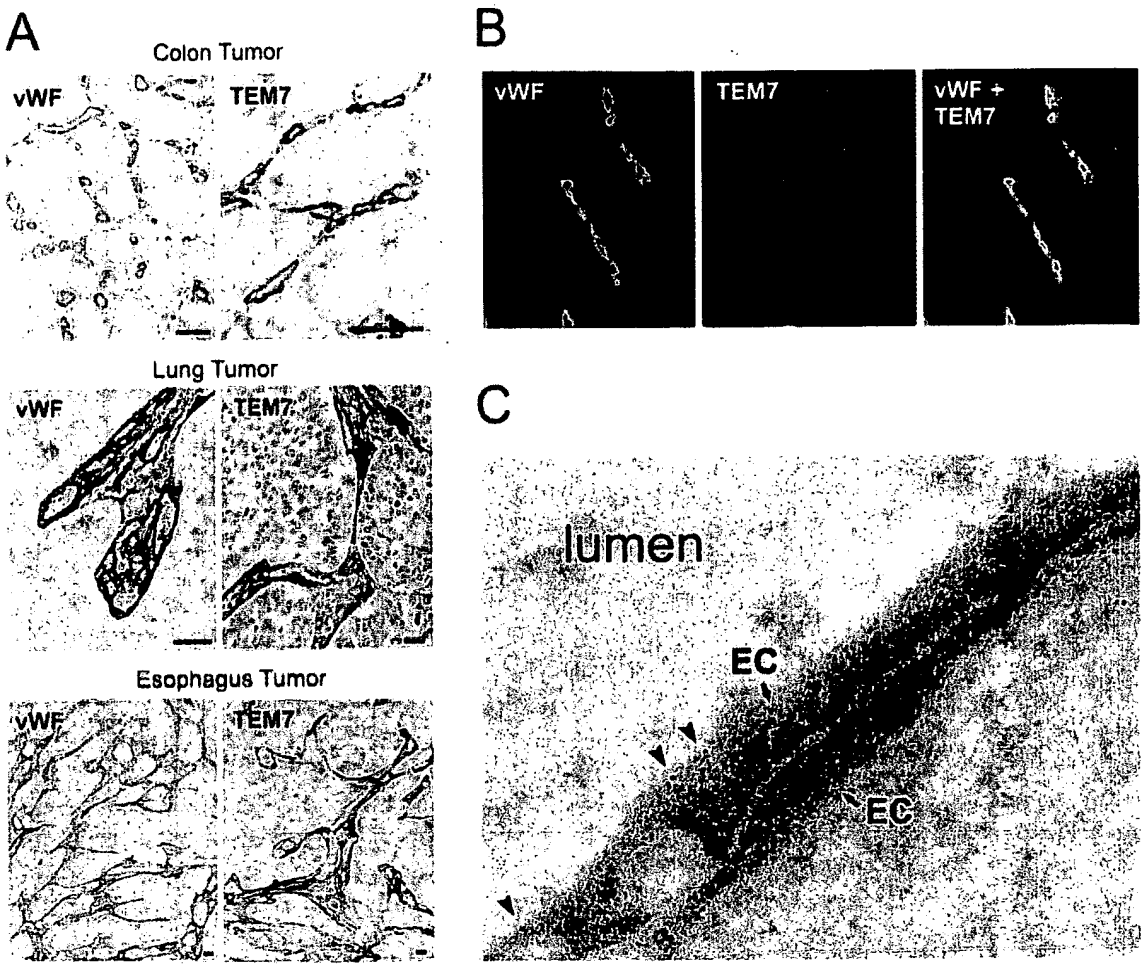
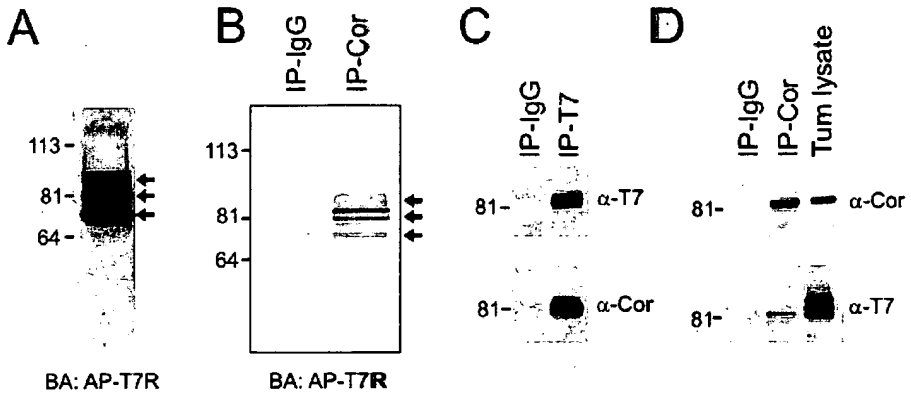
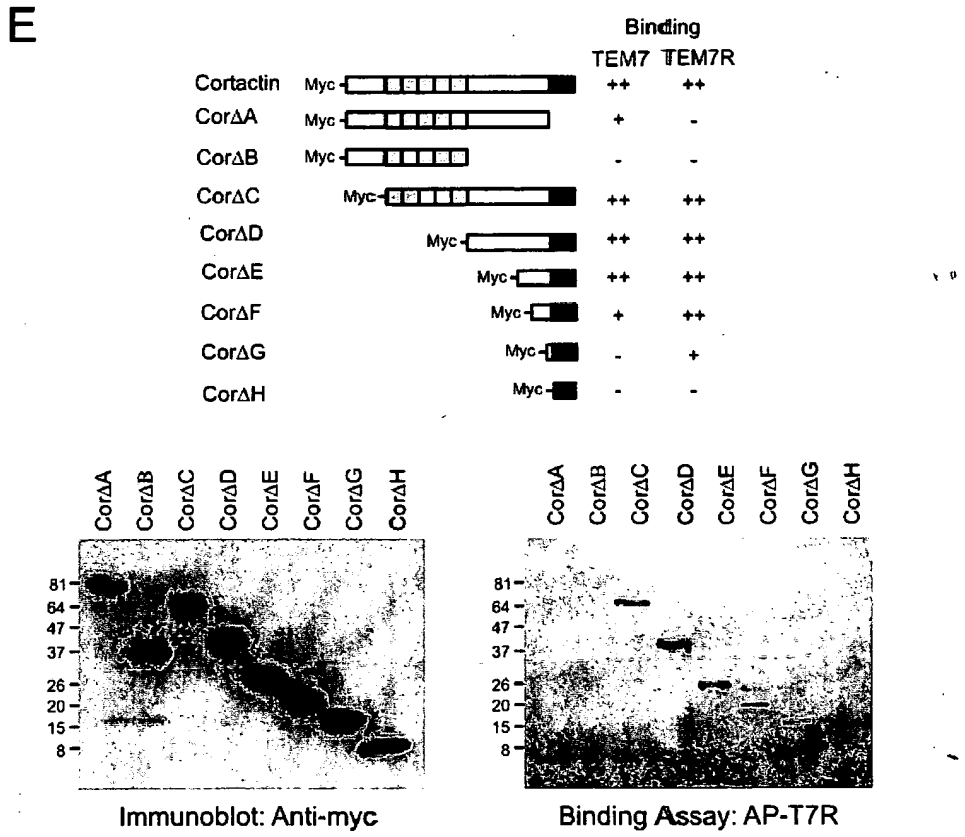


Figure 2, Nanda et al.





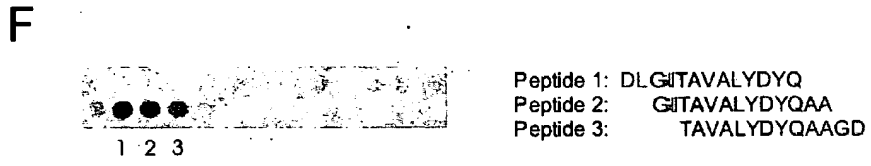


Figure 3, Nanda et al.